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1. Wang L, et al. Int J Oncol. 1999 Apr;14(4):695-701.

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Bone marrow-derived dendritic cells incorporate and process hydrophobized polysaccharide/oncoprotein complex as antigen presenting cells

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Received January 12, 1999; Accepted February 6, 1999

Abstract. We have previously shown that a novel hydrophobized polysaccharide/oncoprotein complex vaccine can induce immune responses against the HER2/*neu*/*c-erbB2* (HER2) expressing tumors. Bone marrow-derived dendritic cells (DCs), as antigen presenting cells (APCs), are the first candidates for presentation of tumor antigens. The aim of this study was to see whether DCs are able to elicit antigen specific host immune responses by stimulating the proliferation of T cells after exposure to cholesteryl group bearing pullulan (CHP) and HER2 protein complex. Vaccination by CHP-HER2 complex was as effective as cholesteryl group bearing mannan (CHM) and HER2 complex on which we reported previously. Immunization of mice with HER2 expressing CMS17HE tumor cells generated both CD4⁺ T cells and CD8⁺ T cells reactive with CHP-HER2 complex pretreated DCs. In addition, immunization with either CHP-HER2 complex or HER2

protein alone could also generate both CD4⁺ T cells and CD8⁺ T cells specifically reactive with CHP-HER2 complex pretreated DCs. The complete rejection of tumors occurred when immunization with CHP-HER2 complex pretreated DCs was started 10 days after tumor inoculation. Therefore, bone marrow-derived DCs pretreated with hydrophobized polysaccharide/oncoprotein complex are a powerful tool for enhancing the effectiveness of oncoprotein for anti-tumor vaccination, opening new options for immune cell therapy.

Introduction

Recently new advances allow the realization of potent vaccination schemes: characterization of tumor antigens (1-3), development of molecular delivery systems (4,5) and manipulation of antigen presenting cells (APCs), such as dendritic cells (DCs) (6). The proto-oncogene HER2/*neu*/*c-erbB2* (HER2) is overexpressed in a variety of human cancers such as breast, ovarian, gastric and renal cancers as well as in other tumor entities (7-11). We have demonstrated that HER2 can be an effective target molecule for specific immune responses against HER2⁺ tumor cells in a syngeneic murine system (4,12). We also reported recently a novel hydrophobized polysaccharide/HER2 oncoprotein complex vaccine, which can induce strong cellular and humoral immune responses against HER2 expressing tumor (4). A truncated protein consisting of the 147 N-terminal amino acids of the proto-oncogene HER2 was complexed with hydrophobized polysaccharides, cholesteryl group bearing mannan (CHM) and cholesteryl group bearing pullulan (CHP), to form nanoparticles. In mice immunized with these complexes, HER2 specific CD8⁺ cytotoxic T lymphocytes (CTLs) could be generated and prevented growth of subsequently inoculated HER2 expressing tumors. The CTL

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Abbreviations: CHM, cholesteryl group bearing mannan; CHP, cholesteryl group bearing pullulan; CAB, carbonic anhydrase II; HER2, HER2/*neu*/*c-erbB2*; CTLs, cytotoxic T lymphocytes; DCs, dendritic cells; rmGM-CSF, recombinant murine granulocyte/macrophage-colony stimulating factor; MHC, major histocompatibility complex; APCs, antigen presenting cells; MR, mannose receptor

Key words: dendritic cells, hydrophobized polysaccharides, HER2/*neu*/*c-erbB2* oncoprotein, tumor vaccine

generated by the immunization with CHM-HER2 complex recognized a peptide spanning the positions 63 to 71 of HER2 (HER2 p63), a part of the truncated protein used for vaccination. CD8⁺ T cells played a major role in the effector phase *in vivo* tumor rejection of host vaccinated with CHM-HER2 complexes. These observations strongly suggested that hydrophobized polysaccharide/truncated HER2 oncoprotein complex could be efficiently delivered to the pathway to produce target antigen peptides recognized by CD8⁺ CTLs and their precursors in the context of MHC class I molecules. In addition, mice immunized with CHM-HER2 complexes could produce an extremely high titer of IgG antibodies against HER2 protein indicating a possible activation of helper CD4⁺ T cells. Detailed mechanisms of antigen presentation in animals immunized by hydrophobized polysaccharides/oncoprotein complexes are still unknown. It has been reported that DCs, as professional APCs, could process and present antigen peptides to T cells efficiently, and could induce anti-tumor immunity (13-18).

We therefore questioned in this study whether bone marrow-derived DCs can incorporate CHP-HER2 complex and process antigenic HER2 oncoprotein to present the cognate antigen peptides to both CD4⁺ T cells and/or CD8⁺ T cells to elicit host immune responses against HER2 expressing tumors. We also examined the usefulness of DCs pretreated with CHP-HER2 complex for the purpose of immune therapy.

Materials and methods

Mice. In all experiments, 6 to 8-week-old female BALB/c mice purchased from Shizuoka Animal Laboratory Center (Shizuoka, Japan) were used and maintained at the Animal Center of Mie University School of Medicine, Tsu, Japan.

Tumor cell lines. CMS 7 and CMS17 are 3-methylcholanthrene-induced fibrosarcoma cell lines of BALB/c mouse origin (3). These lines were transfected with full length cDNA of human HER2 and designated CMS7HE and CMS17HE as described (12).

Antibodies. Anti-CD3 (145-2C11), anti-L3T4/CD4 (GK1.5), anti-Lyt2.2/CD8 (19/178), anti-H-2K^d (20-8-4), anti-I-A^d (MKD-6), anti-B220/CD45R (RA3-3A1/6.2) monoclonal antibodies were produced as described (19). Anti-ICAM-1/CD54 (YN1/1.7.4), anti-LFA-1 (KBA), anti-B7-1/CD80 and anti-B7-2/CD86 monoclonal antibodies were purchased from Pharmingen, USA. Anti-DEC-205 (NLDC-145) was a generous gift from Dr Kraal, Leiden, The Netherlands.

Preparation of cholesteryl group-bearing polysaccharide nanoparticles. CHP-108-0.9 was exactly the same as those used in previous work (20,21). Pullulan (MW = 108,000) was substituted by 0.9 cholesteryl moieties per 100 glucose units of pullulan. An appropriate amount of CHP was dissolved in DMSO and dialyzed against PBS (150 mM, pH 7.9). After dialysis, the suspension was sonicated using a probe type sonifier (TOMY, UR-200P, Tokyo, Japan) at 40 W for 10 min. The obtained suspension was filtered through three types of membrane filters (Super Acrodisc 25, Gelman Science,

pore size: 1.2 mm, 0.45 mm, and 0.2 mm) to make the particles and to remove dust. Finally, an optically clear suspension was obtained. The cholesteryl group-bearing polysaccharides formed nanoparticles by self-aggregation in diameter of 20-30 nm (20,21).

Preparation of complexes between HER2 protein and cholesteryl group-bearing polysaccharides. The HER2 derived protein described above was dissolved in 6 M urea. The protein solution (2.0 mg/ml) was mixed with 2.1 ml of a suspension of cholesteryl-bearing polysaccharides (5.7 mg/ml) at room temperature, resulting in the formation of a CHP-HER2 complex (CHP: 5.0 mg/ml, protein: 0.25 mg/ml, 0.75 M urea) (6,7). CHP-carbonic anhydrase II (CAB, Sigma) complexes were prepared as control using the same method (20-22).

Preparation of T cells and DCs. BALB/c mice were subcutaneously immunized twice with CHP-HER2 complexes (20 µg of truncated HER2 protein and 400 µg of CHP) or three times with mitomycin C treated CMS17HE (2x10⁶) at one week interval. Spleen cells were obtained one week after the last immunization. For preparation of T cell subpopulations, spleen cells were enriched by using nylon fiber columns (23) followed by the treatment with anti-Lyt2.2 (CD8) mAb or anti-L3T4 (CD4) mAb and low toxicity rabbit complement (Cedarlane, Ontario, Canada) to obtain CD4⁺ T cells and CD8⁺ T cells, respectively. Bone marrow-derived DCs were prepared from normal BALB/c bone marrow as described by Inaba *et al.* (24) with minor modifications. Briefly, single bone marrow cell suspensions were obtained from femurs and tibias, then depleted from lymphocytes, granulocytes and Ia⁺ cells by using a mixture of mAbs (anti-CD4, anti-CD8, anti-B220/CD45R and anti-Ia) for 45 min on ice, followed by an incubation with low toxicity rabbit complement for 30 min at 37°C. Cells were resuspended at a concentration of 10⁶ cells/ml of RPMI 1640 medium supplemented with 10 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (rmGM-CSF) and were plated at 3 ml per well of 6-well plates. Floating cells were removed on day 3 and day 5 of culture by gentle pipetting and fed with fresh medium. On day 7 of culture, non-adherent and slightly adherent cells were collected for experiments. The phenotype of DCs were analyzed by FACScan flow cytometry.

T cell proliferation assay. Nylon fiber-purified suspensions of CD4⁺ T cells or CD8⁺ T cells from immunized mice were plated into 96-well U bottom microtiter plates at 3x10⁵ cells/well and used as responder cells. DCs pretreated with CHP-HER2 complex, CHP-CAB complex or HER2 protein only (75 µg protein/ml medium) for 3 h, or untreated DCs were added as stimulator cells at R:S ratio of 40:1 followed 18 h culture. After incubation in RPMI 1640 supplemented with 10% fetal calf serum and 5x10⁻⁵ M 2-mercaptoethanol at 37°C in 5% CO₂ atmosphere for 90 h, cells were labelled with 1 µCi/well ³H-thymidine during the last 18 h of culture and proliferation was determined by microplate scintillation counter. Results are presented as the mean of duplicate (25,26).

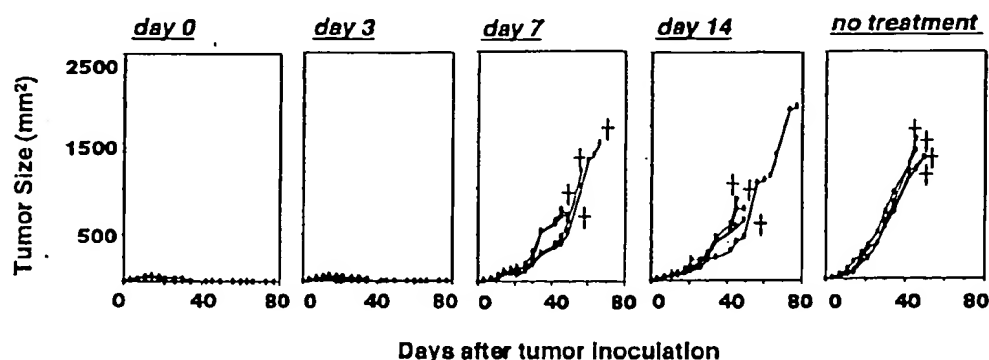


Figure 1. Therapeutic effect of the CHP-HER2 vaccine. BALB/c mice were challenged with 2×10^6 CMS7HE subcutaneously and weekly given CHP-HER2 complex containing 20 μ g of protein starting on the day of challenge, or 3, 7 or 14 days later. Each group consisted of four mice, a line represents a single mouse.

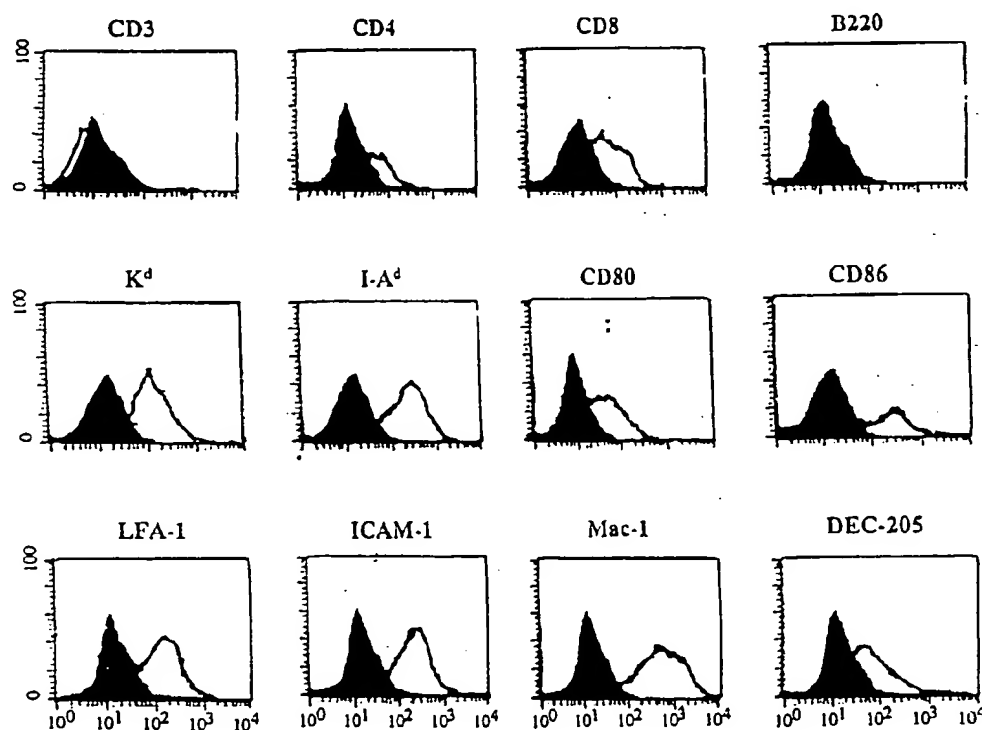


Figure 2. The phenotype of DCs. Bone marrow cells were depleted with anti-CD4, anti-CD8, anti-I-A^d and anti-B220/CD45R, thereafter cultured in the presence of rmGM-CSF (1000 unit/ml). On day 7, the culture cells were harvested and stained with the indicated monoclonal antibodies. The filled histograms represent the isotype controls. The figure shows the results of a representative experiment.

Results

Immunization with the CHP-HER2 complex is therapeutically effective against HER2 expressing tumors. BALB/c mice inoculated with 2×10^6 HER2 expressing CMS7HE tumor cells were given weekly immunization of 20 μ g protein of CHP-HER2 complex starting on the day of the challenge or 3, 7, or 14 days after the tumor challenge, respectively. Complete tumor rejection was observed when the immunization was initiated either on the day of tumor challenge or on day 3 after primary tumor challenge (Fig. 1).

When the immunization was started 7 days or 14 days after the tumor inoculation, only marginal suppression of tumor growth was observed without complete rejection.

DCs can incorporate CHP-HER2 complex and specifically stimulate CD8⁺ T cells and CD4⁺ T cells. We questioned whether bone marrow-derived DCs could incorporate CHP-HER2 complex and stimulate T cells by providing the cognate target peptides. Bone marrow-derived DCs were prepared by culturing bone marrow cells in the presence of rmGM-CSF as described in Materials and methods. The phenotypic

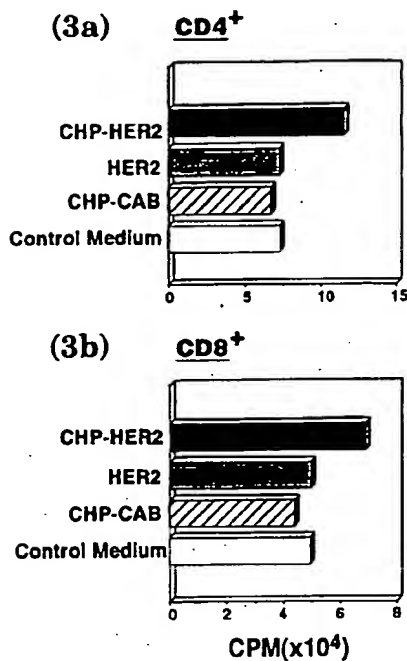


Figure 3. Proliferation of spleen T cells evaluated by ³H-TdR uptake assay. BALB/c mice were immunized subcutaneously three times weekly with 2×10^6 CMS17HE. Responder CD4⁺ T cells and CD8⁺ T cells were prepared from BALB/c mice spleen cells one week after the last immunization. DCs cultured with CHP-HER2 complex, CHP-CAB control complex, HER2 alone or control medium were used as APCs at R:S ratio of 40:1. Both CD4⁺ T cells (a) and CD8⁺ T cells (b) showed the strongest response to DCs treated with CHP-HER2 complex.

characteristics of generated DCs are presented in Fig. 2. BALB/c mice were immunized 3 times with subcutaneous injection of 2×10^6 mitomycin C treated CMS17HE at a weekly interval. One week after the last immunization, CD4⁺ T cell and CD8⁺ T cell subpopulations were prepared. DCs cultured with CHP-HER2 complex, a control CHP-CAB complex, HER2 protein alone for 3 h, or untreated DCs were used as antigen presenting cells to stimulate T cells. Both CD4⁺ T cells and CD8⁺ T cells showed a significantly stronger response to DCs treated with CHP-HER2 complex than to DCs treated with CHP-CAB complex or HER2 protein alone, or to DCs without prior treatment (Fig. 3). A similar type of experiment was performed with T cells from BALB/c mice immunized twice with CHP-HER2 complex or HER2 protein alone subcutaneously at a weekly interval. The proliferative response of T cells was examined by stimulating them with DCs pretreated with CHP-HER2 complex, CHP-CAB control complex, HER2 alone, or without treatment. In both groups immunized with CHP-HER2 complex and HER2 protein alone, CD4⁺ T cells displayed the strongest response to CHP-HER2 pretreated DCs (Fig. 4a and b), similar to the results in CMS17HE immunized mice (Fig. 3). CD4⁺ T cells also responded moderately to DCs pretreated with HER2 protein alone when compared to DCs pretreated with CHP-CAB complex or without treatment. In contrast, CD8⁺ T cells, whether derived from CHP-HER2 complex immunized animals or HER2 immunized animals, responded only to DCs pretreated with CHP-HER2 complex (Fig. 4c and d). These results clearly show that DCs can incorporate CHP-HER2 complexes efficiently and present cognate peptides to both CD4⁺ T cells and CD8⁺ T cells after appropriate processing.

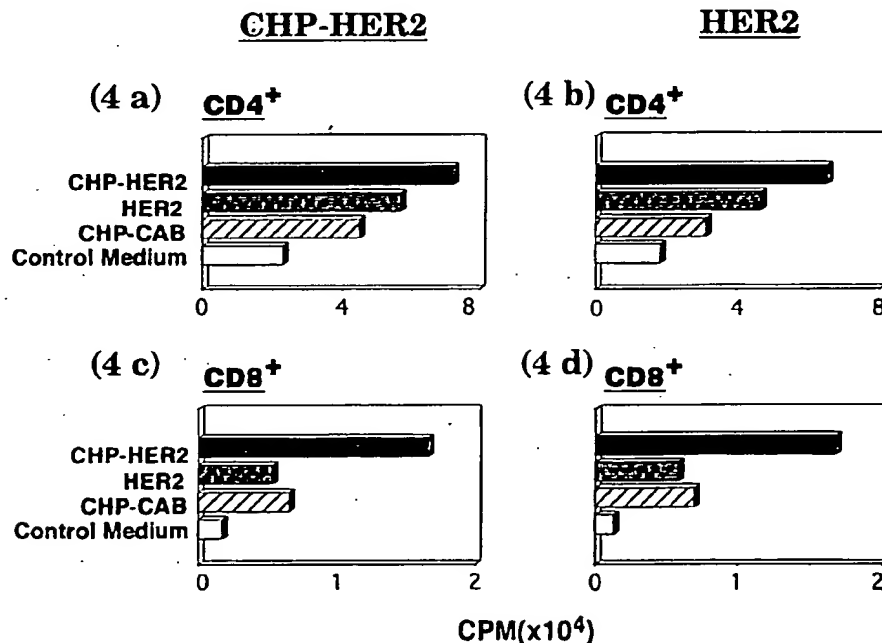


Figure 4. Bone marrow derived DCs demonstrate a potent APC function. DCs pretreated with CHP-HER2 complex, CHP-CAB complex, HER2 alone, or control medium were used as stimulator cells. Responder CD4⁺ T cells and CD8⁺ T cells were obtained from nylon wool-purified spleen cells of mice immunized with CHP-HER2 complex (a and c) or HER2 protein (b and d). ³H-TdR proliferation assay was performed. CD4⁺ T cells (a and b) showed the strongest response to CHP-HER2 complex pretreated DCs, whereas CD8⁺ T cells (c and d) significantly responded only to CHP-HER2 complex pretreated DCs.

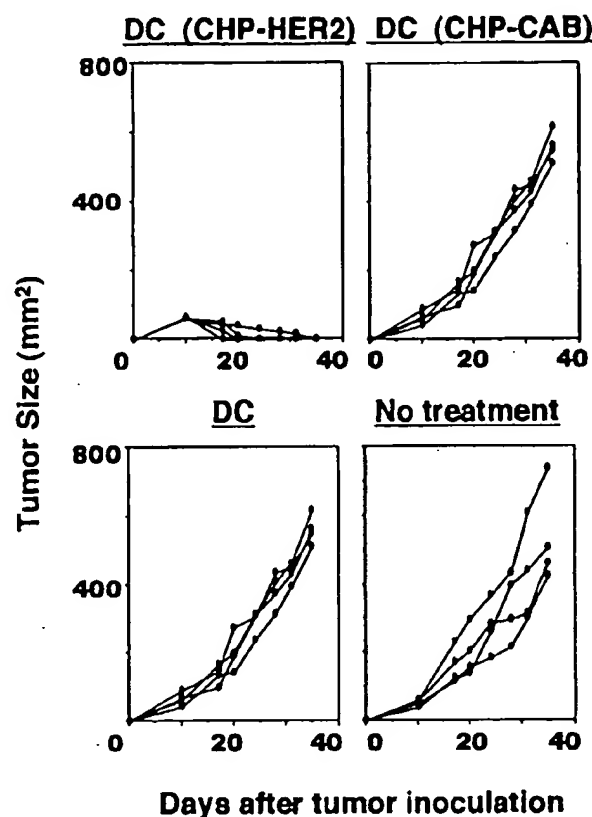


Figure 5. The therapeutic effect of DCs pretreated with CHP-HER2 complex. BALB/c mice were challenged with 2×10^5 CMS7HE subcutaneously. Vaccination with 4×10^5 DCs pretreated with CHP-HER2 complex, CHP-CAB control complex or without prior treatment was started on day 10 after tumor challenge and continued on a weekly basis. Strong tumor suppression was only observed in the group of mice vaccinated with CHP-HER2 complex pretreated DCs. Four mice were used for each experimental group, a line represents a single mouse.

Experimental cell therapy using DCs pretreated with CHP-HER2 complex. We further examined whether DCs treated *ex vivo* with CHP-HER2 complex could be used as vaccine against HER2 expressing tumor cells. 2×10^6 CMS7HE cells were inoculated subcutaneously into BALB/c mice. 10 days after inoculation, vaccination with 4×10^5 DCs pretreated with CHP-HER2 complex or CHP-CAB control complex, or DCs without treatment was started subcutaneously at a weekly basis. As shown in Fig. 5, in the group of mice vaccinated with CHP-HER2 complex pretreated DCs, complete eradication of tumor was observed in all the 4 mice. In contrast, tumor growth in mice of groups either treated with DCs pretreated with CHP-CAB control complex or DCs alone was similar to the tumor growth observed in mice without vaccination.

Discussion

An exogenous soluble protein antigen, when administered to hosts, is in general inefficient in inducing CD8⁺ CTL, since it hardly enters the MHC class I pathway being rather internalized into endosomes and MHC class II presented

(27-32). We reported that soluble truncated hybrid protein of gag and env of human T lymphotropic virus type 1 could induce specific CD8⁺ T cell-dependent immunity when reconstituted into mannan derivative-coated liposomes (30,33). Considering the increasing evidence for receptors that can specifically bind polysaccharide on antigen presenting cells such as DCs and macrophages, we designed a novel and simple protein delivery system by utilizing CHM or CHP complexed with a truncated HER2 protein containing the peptide HER2 p63 that can induce CD8⁺ CTLs against HER2 expressing tumor cells with K^d restriction. We demonstrated that the truncated protein containing 147 N-terminal amino acids of HER2 complexed with CHM or CHP can induce HER2 p63 specific anti-tumor CD8⁺ T cells (4). In the present study we primarily questioned whether bone marrow-derived DCs can incorporate CHP-HER2 complex and process the molecules in order to present the cognate target epitopes to T cells. In addition we questioned whether thus processed DCs are a useful tool for immunotherapy of HER2 expressing tumors.

The CHP-HER2 complex was revealed to be as therapeutically potent as the CHM-HER2 complex on which we reported previously (4). Mice *in vivo* immunized by HER2 expressing CMS17HE tumor cells can generate both CD4⁺ T cells and CD8⁺ T cells specifically reactive with DCs pretreated with CHP-HER2 complex. When mice were immunized with either CHP-HER2 complex or HER2 protein alone, they were also able to generate both CD4⁺ T cells and CD8⁺ T cells specifically reactive with DCs pretreated with CHP-HER2 complex. It is interesting that for these animals either immunized with CHP-HER2 complex or HER2 protein alone, DCs pretreated with CHP-HER2 complex but not HER2 protein alone could strongly stimulate specific CD8⁺ T cells *in vitro* as shown in Fig. 4. These results clearly show that DCs, whether pretreated with CHP-HER2 complex or HER2 protein alone can incorporate and process the antigen peptides and finally present them to CD4⁺ T cells. However, they incorporated and processed antigenic protein in order to sufficiently present the cognate peptides to CD8⁺ T cells only when they were pretreated with CHP-HER2 complex but not with HER2 protein alone. These results support our previous findings that a hydrophobized polysaccharide complexed with antigenic protein could efficiently generate CD8⁺ T cell-dependent immunity either measured *in vitro* as CTL activity or *in vivo* as tumor rejection. The exact molecular mechanisms of how hydrophobized polysaccharide protein complexes are MHC class I presented, remains to be elucidated. Carbohydrate receptors on the cell surface of antigen presenting cells are one of possible explanations. DEC-205 and mannose receptor (MR) probably accept various polysaccharides as binding partners (34,35), that are structurally similar. Both tested substances mannan (4) and pullulan may fit to the binding sites of DEC-205 and/or MR. Since DEC-205 positive (Fig. 2) DCs were cultured and thereafter pulsed with CHP-HER complex, the binding and internalization of the complex is much more probable in our vaccination protocol than in the case of the administration of soluble protein only, which constitutes the beneficial effect for antigen presentation with DCs. The current analysis clearly indicates that there might be antigen

peptide(s) to be recognized by CD4⁺ T cells, in addition to a K^d binding HER2 p63 peptide recognized by CD8⁺ T cells (12) in this truncated HER2 protein. This notion is also supported by the evidence in our previous report that immunization with CHM-HER2 complex could elicit extremely high titers of IgG antibodies against the truncated HER2 protein suggesting an indispensable role of CD4⁺ helper T cells (4). The characterization of the precise amino acid sequence of the peptide recognized by CD4⁺ T cells is ongoing.

Having established that bone marrow-derived DCs can efficiently stimulate both CD4⁺ T cells and CD8⁺ T cells (13-18), we examined their usefulness for immunotherapy of HER2 expressing tumors. As shown in Fig. 5, treatment of mice inoculated with CMS7HE 10 days prior to immunization, obvious suppression of tumor growth was observed in the group utilizing DCs pretreated with CHP-HER2 complex. Non-specific adjuvant effect of CHP seems to be unlikely because DCs treated with CHP-CAB control complex showed no effect for tumor suppression when compared with mice without any immunization. It is of particular interest that in mice immunized with CHP-HER2 complex pretreated DCs, there was complete tumor eradication observed in all the 4 mice. In our experience, immunization either with CHP-HER2 complex or CHM-HER2 complex, complete tumor suppression was possible only when we initiated it sooner than 4 days following tumor inoculation. The present data strongly suggest that CHP-HER2 complex can be effectively used as a cancer vaccine in concert with bone marrow-derived DCs for immunological cell therapy.

Acknowledgments

This work was supported in part by grants of Scientific Research on Priority Areas (A) from the Ministry of Education, Science, Sports and Culture (MONBUSHO) of Japan. L-J Wang is a recipient of a fellowship from the MONBUSHO. We thank Ms. Seiko Lanaway for expert secretarial work and Ms. Miwa Usui and all other colleagues who provided excellent assistance and useful information.

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L6: Entry 4 of 6

File: USPT

Dec 8, 1998

US-PAT-NO: 5846538

DOCUMENT-IDENTIFIER: US 5846538 A

TITLE: Immune reactivity to HER-2/neu protein for diagnosis and treatment of malignancies in which the her-2/neu oncogene is associated

DATE-ISSUED: December 8, 1998

INVENTOR-INFORMATION:

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US-CL-CURRENT: 424/185.1; 514/2, 530/300, 530/350

CLAIMS:

We claim:

1. A method for treating a malignancy in a human, wherein a HER-2/neu oncogene is associated with the malignancy, comprising immunizing a human with a HER-2/neu peptide recognized by T cells, said peptide not being the extracellular domain of the protein expression product of a HER-2/neu oncogene.
2. The method of claim 1 wherein a HER-2/neu oncogene is associated with a malignancy selected from the group consisting of breast, ovarian, colon, lung and prostate cancer.
3. The method of claim 1 wherein the step of immunizing comprises administering the HER-2/neu peptide repetitively to the human.
4. The method of claim 1 wherein the peptide has the amino acid sequence of FIG. 1 from lysine, amino acid 676, to valine, amino acid 1255 (SEQ ID NO: 69).
5. A method for eliciting or enhancing in a human an immune response to the protein expression product of a HER-2/neu oncogene, comprising immunizing a human with a HER-2/neu peptide recognized by T cells, said peptide not being the extracellular domain of the protein expression product of a HER-2/neu oncogene.
6. The method of claim 5 wherein the peptide has the amino acid sequence of FIG. 1 from lysine, amino acid 676, to valine, amino acid 1255 (SEQ ID NO: 69).

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L2: Entry 1 of 1

File: USPT

Jun 13, 2000

DOCUMENT-IDENTIFIER: US 6075122 A

TITLE: Immune reactivity to HER-2/neu protein for diagnosis and treatment of malignancies in which the HER-2/neu oncogene is associated

US Patent No. (1):
6075122Brief Summary Text (15):

Within a related aspect, the present invention provides anti-cancer therapeutic compositions comprising T cells proliferated in the presence of HER-2/neu protein, in combination with a pharmaceutically acceptable carrier or diluent. In addition, a variety of peptides designated for CD8^{sup}.+ T cell responses are provided which include peptides consisting essentially of:

Detailed Description Text (19):

Regardless of how an individual's T cells are proliferated in vitro, the T cells may be administered to the individual as an anti-cancer composition in an amount effective for therapeutic attack against a tumor. Thus, a patient's own T cells (autochthonous T cells) can be used as reagents to mediate specific tumor therapy. Typically, about 1.times.10^{sup.9} to 1.times.10^{sup.11} T cells/M^{sup.2} will be administered intravenously or intracavitary, e.g., in pleural or peritoneal cavities, or in the bed of a resected tumor. It will be evident to those skilled in the art that the number and frequency of administration will be dependent upon the response of the patient. Pharmaceutically suitable carriers or diluents for T cells include physiological saline or sera. It will be recognized by one skilled in the art that the composition should be prepared in sterile form.

Detailed Description Text (21):

In addition to the HER-2/neu peptide (which functions as an antigen), it may be desirable to include other components in the vaccine, such as a vehicle for antigen delivery and immunostimulatory substances designed to enhance the protein's immunogenicity. Examples of vehicles for antigen delivery include aluminum salts, water-in-oil emulsions, biodegradable oil vehicles, oil-in-water emulsions, biodegradable microcapsules, and liposomes. Examples of immunostimulatory substances (adjuvants) include N-acetylmuramyl-L-alanine-D-isoglutamine (MDP), lipopoly-saccharides (LPS), glucan, IL-12, GM-CSF, gamma interferon and IL-15. It will be evident to those skilled in this art that a HER-2/neu peptide may be prepared synthetically or that a portion of the protein (naturally-derived or synthetic) may be used. When a peptide is used without additional sequences, it may be desirable to couple the peptide hapten to a carrier substance, such as keyhole limpet hemocyanin.